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Application of: Martin E. Schwab *et al.*

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For: NUCLEOTIDE AND PROTEIN SEQUENCES OF NOGO GENES AND METHODS BASED THEREON

Attorney Docket No.: 10200-003-999

DECLARATION OF PROF. DR. MARTIN E. SCHWAB UNDER 37 C.F.R. § 1.132

Sir:

I, Martin E. Schwab, of Zurich, Switzerland, do declare and state that:

(1) I am a citizen of Switzerland residing at Berninastrasse 96, Zurich, Switzerland, CH-8057.

(2) I am a co-inventor with Maio S. Chen of the invention described and claimed in the above-identified patent application.

(3) I am currently Professor of Neuroscience at the Brain Research Institute at the University of Zurich, the assignee of the present application.

(4) My academic and technical experience and honors, and a list of my publications are set forth in my *curriculum vitae,* which is attached hereto as Exhibit 1.

(5) The above-identified application discloses and claims, *inter alia*, Nogo proteins and related proteins that are free of all central nervous system ("CNS") myelin.

CARONI-1

(6) I am a co-author of the paper entitled "Two Membrane Protein Fractions from Rat Central Myelin with Inhibitory Properties for Neurite Growth and Fibroblast Spreading" by Caroni and Schwab, 1988, Journal of Cell Biology, 106:1281-1288 ("Caroni-1," attached

as Exhibit 2). I am familiar with the experiments described in Caroni-1.

(7) In Caroni-1, we describe attempts to purify by conventional biochemical techniques the proteins that are responsible for the inhibitory substrate effect of rat CNS myelin on neurite outgrowth. Rat CNS myelin material was solubilized in sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) sample buffer and the myelin components were separated by SDS-PAGE (page 1285, left column, third paragraph). Subsequently, myelin proteins were extracted from gel regions corresponding to the migration distance of 35 kD and 250 kD proteins (page 1285, right column, first paragraph). As stated at page 1285 of Caroni-1 (the paragraph spanning the left and right columns and Figure 4), two protein fractions, namely a 35 kD fraction and a 250 kD fraction, with neurite outgrowth inhibitory activity were obtained by fractionating CNS myelin material using this purification procedure.

(8) The 35 kD fraction and the 250 kD fraction resulting from the purification procedure of Caroni-1 were contaminated with central nervous system myelin material. As stated at page 1285 of Caroni-1, right column, lines 8-9, the regions of the SDS-PAGE gel from which these protein fractions were extracted contained more than one protein species.

(9) We later attempted to use High Performance Liquid Chromatography (HPLC) to isolate individual proteins from the 35 kD fraction and 250 kD fraction for protein microsequencing. In the years between 1992 and 1994, we obtained short peptide sequences from the HPLC fractions. However, the peptide sequences obtained were only short and of insufficient quality to allow us to identify the novel protein. None of these peptide sequences obtained at that time from the HPLC fractions corresponded to the amino acid sequence of Nogo protein, which was determined later after the Nogo gene had been cloned. Thus, the HPLC fractions were of insufficient purity and contained a majority of myelin proteins other than Nogo protein. Thus, even if the HPLC purified material contained Nogo protein, any such Nogo protein could not have been free of all CNS myelin material.

(10) Since the HPLC-purified material contained myelin proteins other than Nogo protein, the 35 kD and the 250 kD protein fractions from which the HPLC-purified material was obtained also contained myelin proteins other than Nogo protein.

CARONI-2

(11) I am a co-author of the paper entitled "Antibody against Myelin-Associated Inhibitor of Neurite Growth Neutralizes Nonpermissive Substrate Properties of CNS White Matter" by Caroni and Schwab, 1998, Neuron 1:85-96 ("Caroni-2," attached as Exhibit 3). I am familiar with the experiments described in Caroni-2.

(12) We generated monoclonal antibodies against the 250 kD fraction and the 35 kD fraction, respectively, described in Caroni-1. The monoclonal antibodies were termed IN-1 and IN-2 (see Abstract of Caroni-2). We attempted to use IN-1 in an immunoprecipitation to isolate inhibitory proteins from CNS myelin using two different protocols (see Caroni-2, pages 89-91, and 95 (third paragraph); legend of Table 4). Briefly, in Protocol 1, solubilized CNS myelin protein was incubated with IN-1. In Protocol 2, IN-1 antibody was incubated with intact myelin membranes, and antigen-antibody complexes were subsequently solubilized. In both protocols, rabbit anti-mouse antibodies and *S. aureus* cells were used to sediment the antigen-antibody complexes. Dissociation of the antigen-antibody complexes that were obtained using both protocols, by elution of the antigen, acetone precipitation, and reconstitution into liposomes, yielded proteins with inhibitory activity. Additionally, IN-1 immunoprecipitated protein from Protocol 2 was separated by SDS-PAGE and gel regions corresponding to 35 kD and 250 kD molecular weights were extracted and reconstituted into liposomes (Table 4 and legend of Table 4, at page 91).

(13) The material immunoprecipitated by IN-1 and recovered in Protocol 1 and Protocol 2 was not free of all CNS myelin material. When we conducted Western blot analysis of CNS myelin material using IN-1 (see Figure 5 of Caroni-2), IN-1 reactive bands of molecular weights other than the molecular weights of 35 kD and 250 kD were observed. Moreover, when proteins immunoprecipitated by IN-1 were separated by SDS-PAGE, multiple protein bands of molecular weights other than the molecular weights of the 35 kD and the 250 kD fractions were observed. Thus, the material that was immunoprecipitated with IN-1 antibody contained myelin proteins other than Nogo protein because of the ability of IN-1 to bind to proteins other than Nogo protein.

(14) IN-1 is an IgM antibody. The material that was obtained by immunoprecipitation using IN-1 and subsequent gel extraction could not be expected to be pure using an IgM as the precipitating antibody, since IgM antibodies are commonly known

to display nonspecific binding (see, *e.g.*, page 56, second full paragraph, of Epstein, 1994, FDA Regulation of HIV-Related Tests and Procedures, In: AIDS Testing-A Comprehensive Guide to Technical, Medical, Social, Legal, and Management Issues, Eds. Schochetman and George, 2nd edt., Springer, New York; attached as Exhibit 4). The material was not free of all CNS myelin material as later shown in my laboratory by SDS-PAGE analysis (data not shown in Caroni-2).

(15) In Caroni-2, IN-2 was shown to bind to CNS myelin inhibitory substrates and to neutralize their non-permissive substrate properties (Caroni-2, at page 89, left column, and Table 3 at page 91). IN-2 was not used in immunoprecipitation experiments in Caroni-2. Caroni-2 shows that IN-2 also recognizes proteins other than inhibitory proteins, such as cytoskeleton-associated antigens from astrocytes (Caroni-2, at page 89, left column). In subsequent experiments in my laboratory, we observed that IN-2 reacts with intermediate filaments (glial fibrillary acidic protein (GFAP) and neurofilaments in the CNS). In a Western blot analysis of CNS material that had been separated by one- or two-dimensional SDS-PAGE, the IN-2 antibody detected bands/spots of the size and charge of neurofilament and GFAP (B. Rubin, PhD Thesis, University of Zurich, 1995). Thus, immunoprecipitates of CNS myelin material using IN-2 would be contaminated with other proteins such as neurofilament.

SPILLMANN

(16) I am a co-author of the paper entitled "High Molecular Weight Protein of Human Central Nervous System Myelin Inhibits Neurite Outgrowth: an Effect which can be Neutralized by the Monoclonal Antibody IN-1" by Spillmann *et al.*, 1997, European Journal of Neuroscience, 9:549-555 ("Spillmann," attached as Exhibit 5). I am familiar with the experiments described in Spillmann.

(17) In Spillmann, we describe attempts to purify by conventional biochemical techniques the proteins that are responsible for the inhibitory substrate effect of human CNS myelin on neurite outgrowth. CNS myelin material was solubilized and the solubilized proteins were subsequently separated using SDS-PAGE (see sections "Preparation of myelin proteins" and "Gel analysis of inhibitory proteins from CNS myelin" at page 550 of

Spillmann). Proteins were eluted from the 0-100, 100-200, and 200-300 kD regions of the SDS-PAGE gel (see section "Gel analysis of inhibitory proteins from CNS myelin" at page 550 of Spillmann). As stated at page 552 of Spillmann, the paragraph spanning the left and right columns, and the legend to Figure 3, only the protein fraction of proteins with molecular weights between 200 and 300 kD displayed neurite-outgrowth inhibitory activity.

(18) The 200 kD to 300 kD fraction was contaminated with CNS myelin material. The proteins were obtained by eluting all CNS myelin proteins from the 200 kD to 300 kD region of an SDS-PAGE gel. Thus, numerous human CNS myelin proteins with a molecular weight between 200 kD and 300 kD should be present in this fraction. Any Nogo protein present in this fraction was therefore not free of all CNS myelin material.

(19) Later attempts to sequence the proteins in the 200 kD to 300 kD fraction obtained by the above procedure failed to identify a Nogo protein due to the high level of contamination with CNS myelin proteins other than Nogo protein.

CHEN-1

(20) I am a co-author of the abstract entitled "Molecular Cloning of a Gene for a Putative Inhibitor to CNS Regeneration" by Chen *et al.* for the 27th Annual Meeting of the Society for Neuroscience, 1997 ("Chen-1;" attached as Exhibit 6). This abstract accompanied a poster that was presented at the 27th Annual Meeting of the Society for Neuroscience. I do not have in my possession a copy of the poster. I am familiar with the experiments described in Chen-1.

(21) Chen-1 lists three cDNA clones: EST(rat), Oli18(rat), and CWP1-3(bovine). Chen-1 also states that, based on Northern blot analyses, the gene corresponding to these cDNA clones likely encodes three different transcripts. These cDNA clones were later shown to represent partial sequences of the Nogo gene.

(22) Chen-1, and the related poster, did not disclose any nucleotide or protein sequences of Nogo. Without this sequence information, it would have required extensive experimentation to obtain the nucleic acids that encode the Nogo proteins. For example, to employ the method suggested by Chen, first, it would have been necessary to obtain Nogo protein pure enough for protein sequencing. Second, degenerate oligonucleotides would have

had to be designed based on the protein sequence to screen cDNA libraries.

(23) The first step listed in paragraph 22 requires more than routine experimentation. As evidenced by my discussion of Caroni-1, Caroni-2, and Spillmann hereinabove, conventional biochemical techniques are insufficient to obtain Nogo protein pure enough for protein sequencing. In fact, it took almost ten years for my laboratory to develop a purification procedure capable of yielding sequencing-grade Nogo protein, and I am aware that several competing laboratories failed in their attempts to purify Nogo protein.

(24) Successful implementation of the second step listed in paragraph 22 to obtain a Nogo cDNA also requires more than routine experimentation. Because the oligonucleotide sequences that are designed based on the peptide sequences are degenerate, only a small subset of oligonucleotides will be complementary to Nogo cDNA sequences. Consequently, detection of a cDNA in a cDNA library using these degenerate probes is more difficult and less predictable than screening of a cDNA library using traditional probes. When my laboratory ultimately carried out this method, we had to perform several rounds of cDNA screens to obtain the full length cDNA because of the considerable length of the Nogo gene. In fact, we had to use several partial cDNA sequences to assemble the full length cDNA. In addition, it proved very difficult to obtain cDNA clones encoding the amino terminal portion of Nogo A and Nogo B. To overcome these difficulties, we used the sequences of the originally isolated cDNA clones that encoded the carboxy terminal portion of Nogo to search for EST sequences in a publicly available database. Subsequently, we used the EST sequences to design new probes to perform additional cDNA screens. This strategy allowed us finally to obtain cDNA clones encoding the amino terminal portion of Nogo A and Nogo B proteins. Thus, it would have taken extensive experimentation based upon the disclosure of Chen-1 to obtain full-length cDNA sequences encoding rat and/or human protein.

(25) I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Oct. 2nd 2006

Martin E. Schwab
MARTIN E. SCHWAB

Attachments:

Exhibit 1:	*Curriculum Vitae* of Martin E. Schwab
Exhibit 2:	Caroni and Schwab, 1988, Journal of Cell Biology, 106:1281-1288;
Exhibit 3:	Caroni and Schwab, 1998, Neuron 1:85-96;
Exhibit 4:	Epstein, 1994, FDA Regulation of HIV-Related Tests and Procedures, In: AIDS Testing-A Comprehensive Guide to Technical, Medical, Social, Legal, and Management Issues, Eds. Schochetman and George, 2nd edt., Springer, New York
Exhibit 5:	Spillmann *et al.*, 1997, European Journal of Neuroscience, 9:549-555; and
Exhibit 6:	Abstract entitled "Molecular Cloning of a Gene for a Putative Inhibitor to CNS Regeneration" by Chen *et al.* for the 27th Annual Meeting of the Society for Neuroscience, 1997